Title: **AI-2 quorum sensing inhibitors affect the starvation response and reduce virulence in several *Vibrio* species, most likely by interfering with LuxPQ**

Running title: AI-2 quorum sensing inhibitors

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Abstract

The increase of disease outbreaks caused by *Vibrio* spp. in aquatic organisms as well as in humans, together with the emergence of antibiotic resistance in *Vibrio* spp., has led to a growing interest in alternative disease control measures. Quorum sensing (QS) is a mechanism for regulating microbial gene expression in a cell-density dependent way. While there is good evidence for the involvement of auto-inducer 2 (AI-2) based interspecies QS in the control of virulence in multiple *Vibrio* spp., only few inhibitors of this system are known. From the screening of a small panel of nucleoside analogues for their ability to disturb AI-2 based QS, an adenosine derivative with a p-methoxyphenylpropionamide moiety at C-3', emerged as a promising hit. Its mechanism of inhibition was elucidated by measuring the effect on bioluminescence in a series of *Vibrio harveyi* AI-2 QS mutants. Our results indicate that this compound, as well as a truncated analogue lacking the adenine base, block AI-2 based QS without interfering with bacterial growth. The active compounds neither affected the bioluminescence system as such, nor the production of AI-2, but most likely interfered with the signal transduction pathway at the level of LuxPQ in *V. harveyi*. The most active nucleoside analogue (designated LMC-21) was found to reduce *Vibrio* spp. starvation response, to affect biofilm formation in *Vibrio anguillarum, Vibrio vulnificus* and *Vibrio cholerae*, to reduce pigment and protease production in *V. anguillarum* and to protect gnotobiotic *Artemia* from *V. harveyi*-induced mortality.

Introduction

Vibrio species are ubiquitous in marine environments worldwide (Igbinosa & Okoh, 2008). As opportunistic pathogens they can cause mild to severe infections in humans and marine animals. Vibriosis is one of the most prevalent fish diseases, mainly caused by *Vibrio anguillarum*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio harveyi*, and *Vibrio campbellii* (Garcia *et al.*, 1997; Austin & Zhang, 2006). Other *Vibrio* spp. are pathogenic for humans. *Vibrio vulnificus* is associated with gastro-intestinal infections primarily following the consumption of raw and undercooked seafood, but it can also cause wound or soft-tissue infections (Bross *et al.*, 2007). In addition, systemic *V. vulnificus* infections are notorious for their high mortality rate (Chiang & Chuang, 2003). *Vibrio cholerae* is responsible for pandemic and epidemic outbreaks of cholera (Griffith *et al.,* 2006). *V. cholerae* serotype O1 causes the majority of the outbreaks worldwide, while the O139 serotype has only been detected in South-East and East Asia (Sack *et al.*, 2004; Griffith *et al.*, 2006). Cell-cell communication (quorum sensing, QS) in *Vibrio* spp. plays an important role in virulence. QS in *Vibrio* spp. involves three types of signal molecules. *N*-acyl-homoserine lactones (AHL) are used in the LuxM/N QS system, cholera auto-inducer 1 (CAI-1) in the CqsA/S and auto-inducer 2 (AI-2) in the LuxS/PQ QS system (Bassler *et al.*, 1993; Bassler *et al.*, 1997; Higgins *et al.*, 2007; Ryan & Dow, 2008). AI-2 is synthesized starting from *S*-adenosylmethionine, which (through a series of enzymatic reactions, including the reaction catalysed by LuxS) is converted to 4,5-dihydroxy-2,3-pentanedione (DPD) (Surette *et al.*, 1999; Winzer *et al.*, 2002). The spontaneous cyclisation of DPD followed by esterification with a tetrahydroxyborate anion results in the formation of AI-2 (Miller *et al.*, 2004). In *Vibrio* spp., sensing of

extracellular AI-2 involves two proteins, LuxP and LuxQ (Chen *et al.*, 2002). At low AI-2 concentration, LuxQ will be autophosphorylated resulting in the transfer of a phosphate group to LuxO via LuxU (Freeman & Bassler, 1999a; Freeman & Bassler, 1999b). Phosphorylation of LuxO leads to its activation and the production of small regulatory RNAs. These small RNAs, together with the chaperone protein Hfq, destabilise mRNA of the response regulator LuxR. In the absence of AI-2, LuxR is not produced and LuxR-dependent genes are not transcribed. Binding of AI-2 to the LuxPQ complex initiates a switch from kinase to phosphatase activity, which results in the dephosphorylation of the downstream proteins LuxU and LuxO. Dephosphorylated LuxO is inactive and does not induce the production of small regulatory RNAs. Hence, the response regulator LuxR is produced and initiates transcription of target genes, including several virulence genes. Therefore, QS inhibitors are promising antipathogenic agents. Due to the presence of the *luxS* gene in diverse bacterial species, AI-2 is considered to be a signal for inter-species communication (Xavier & Bassler, 2003). However, the LuxPQ signal transduction system is restricted to Vibrionales (Sun *et al*., 2004; Rezzonico & Duffy, 2008). The increase of *Vibrio* disease outbreaks in aquatic organisms as well as in humans (Harvell *et al.*, 2002; Boyd *et al.*, 2008; Kapp, 2009), together with the emergence of antibiotic resistance in *Vibrio* spp. (Karunasagar *et al.*, 1994; Scrascia *et al.*, 2006), has resulted in a growing interest in alternative disease control measures (Lynch & Wiener-Kronish, 2008). A novel approach consists of interfering with bacterial communication (Ni *et al.*, 2009). Several cinnamaldehyde and furanone derivatives disrupt AI-2 based QS in *Vibrio* spp. by decreasing the DNA-binding activity of the response regulator LuxR and are active both *in vitro* and *in vivo* (Defoirdt *et al.*, 2006; Defoirdt *et al.*, 2007; Brackman *et*

92 *al.*, 2008). Other compounds, including *S*-anhydroribosyl-L-homocysteine and *S*-
93 homoribosyl-L-cysteine, block the production of AI-2 by inhibiting the key enzyme LuxS
94 (Alfaro *et al.*, 2004; Shen *et al.*, 2006). Based on the concept of molecular mimicry and
95 through virtual screenings using the crystal structure of LuxP, new AI-2 QS inhibitors
96 have previously been discovered (Li *et al*., 2008; Ni *et al*., 2008a; Ni *et al*., 2008b).
97 However, although these compounds affect bioluminescence in *V. harveyi*, they were
98 neither evaluated for their effect on QS-regulated virulence factors, nor for their activity
99 *in vivo*. The goal of the present study was to test whether previously described AI-2 QS
100 inhibitors targeting LuxPQ and various compounds from our collection have the ability to
101 block the production of QS-regulated virulence factors in *Vibrio* spp.
102

Materials and methods

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 1. They were cultured in Marine-Broth (MB) (BD) in the presence of appropriate antibiotics at 30 °C with shaking, except for *Escherichia coli* DH5α and *E. coli* K12, which were grown in Luria-Bertani broth (LB) (BD) at 30 °C and 37 °C, respectively, without shaking.

Compound library

The compounds used in the present study consisted of a selected set of known AI-2 QS inhibitors, supplemented with a series of nucleoside (mainly: adenosine) analogues (Fig. 1). 3'-Azido- (3'-N_3-3'-dA) and 3'-amino-3'-deoxyadenosine (3'-NH_2-3'-dA) have been prepared as reported (Azhayev & Smrt, 1978) and are nowadays also commercially available. For the synthesis of the amide analogues derived from 3'-NH_2-3'-dA (i.e., LMC-23, LMC-20, IK-1, LMC-21, LMC-27 and LMC-28) we followed a procedure that was described before (Soenens *et al.,* 1995). Briefly, the 3'-amino group of unprotected 3'-NH_2-3'-dA was acylated with the appropriate carboxylic acids using dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC) and *N*-hydroxysuccinimide (NHS) as coupling agents in a mixture of DMF and dichloromethane (Supplementary Data, Fig. S1). The synthetic route followed for the synthesis of the 3'-branched-chain analogue SC-23, differing from LMC-21 by the insertion of a CH_2 group between C-3' of the ribofuranose ring and the amide moiety started from the previously described intermediate 1 (Kim *et al.,* 2003) (Supplementary Data, Fig. S2). The 2-modified adenosine analogues LMC-29, LMC-30 and LMC-35 were recently synthesized

and found to be potent adenosine A_3 receptor antagonists/partial agonists (Cosyn *et al.,* 2006), while PVR-121 is an agonist for the same receptor (Ohno *et al.*, 2004). The amides derived from 3-(4-methoxyphenyl)propanoic acid (i.e., SC-1, SC-2 and SC-3) were prepared by EDC-mediated coupling of the parent carboxylic acid with the appropriate amine in the presence of TEA. The synthesis of SC-20 started from the known sugar intermediate 4 (Supplementary Data, Fig. S3) that was converted to methyl glycoside 5 upon reaction with $SnCl_4$ and dry MeOH (Moradei *et al*., 1991). Remarkably, during this reaction a larger amount of 6 was formed. The reaction mixture could be efficiently separated by flash chromatography and 5 and 6 were separately deprotected upon treatment with NH_3 in MeOH, thereby affording 7.1 and 7.2. NMR-analysis revealed that 7.1 and 7.2 only differ at the anomeric position (α- or β-MeO group). Although the configuration of each anomer remains uncertain, we anticipate that 7.1 represents the β-anomer, since it was formed from 5, which still possessed the participating acetate group at C-2. Subsequently, we continued with azide 7.2 (presumably the α-anomer), which was reduced through a Staudinger reduction. Finally, the resulting amine was coupled to 3-(4-methoxyphenyl)propanoic acid using HCTU as the coupling agent. All synthesized compounds were structurally confirmed using ^{1}H- and ^{13}C nuclear magnetic resonance spectroscopy and exact mass measurements (Supplementary Data) and were shown to possess a purity of at least 95% by combustion analysis. The previously described AI-2 QS inhibitor, 2-(2-thienylsulfonyl)ethanethioamide (KM-03009) (Li *et al*., 2008) was purchased from Acros Organics, while pyrogallol (Ni *et al.,* 2008a) and 4-methoxycarbonyl-phenylboronic acid (MCPBA) (Ni *et al.,* 2008b) were purchased from Sigma-Aldrich. If necessary,

compounds were diluted in DMSO (final concentration of 0.5 % v/v). The stock solutions were stored at -20 °C. Control solutions contained the same amount of DMSO.

Determination of the minimal inhibitory concentrations (MIC)

MICs were determined for each compound by using a microdilution assay, as previously described (Brackman *et al*., 2009). MB and LB medium were used for all *Vibrio* spp. and both *E. coli* strains, respectively. The plates were incubated and the absorption at 590 nm was measured after 24 h using a Victor Wallac² multilabel counter (Perkin Elmer Life and Analytical Sciences).

Identification of the molecular target of the QS inhibitors

The assay for the effect on constitutively expressed bioluminescence (using *E. coli* DH5α containing the pBlueLux plasmid), the bioassay for LuxS inhibition (using *V. harveyi* MM30) and assays to determine the molecular target of the compounds tested (using *V. harveyi* BB120, BB170, BB886, JAF375, JAF553, JAF483, JMH597 and BNL258) were conducted as described previously (Brackman *et al*., 2008). Each compound was tested at least six times in triplicate ($n \geq 18$).

Effect on QS regulated virulence phenotypes *in vitro*

The effect of AI-2 QS inhibitors on pigment production and protease activity in *V. anguillarum* LMG 4411 was determined as described previously (Croxatto *et al*., 2002). Each compound was tested at least twice in triplicate ($n \geq 6$). Biofilms were grown according to Brackman *et al.* (2008). In brief, the *Vibrio* strains were grown overnight in

MB and approximately 10^8 colony forming units ml^{-1} was added to the wells of a 96 well microtiter plate in the presence or absence of QSI compounds. Bacteria were allowed to adhere and grow without agitation for 4 h at 30°C. After 4 h, plates were emptied and rinsed with sterile physiological saline (PS). After this rinsing step, fresh MB (with or without compounds) was added and the plate was incubated for 20 h at 30°C.

Biofilm biomass was quantified by crystal violet (CV) staining (Peeters *et al*., 2008). The control signal corresponds to an A_{590} of 0.604 ± 0.108 and 0.639 ± 0.129 for *V. anguillarum* LMG4411 and *V. vulnificus* LMG16867, respectively. For quantification of the number of metabolically active (i.e. living) cells in the biofilm, a resazurin assay was used (Peeters *et al*., 2008). Each compound was tested at least six times in triplicate ($n \geq 18$).

Effect on QS regulated stress responses *in vitro*

Vibrio spp. were grown overnight in MB, cells were collected by centrifugation and resuspendend in artificial seawater (ASW) (Bang *et al.*, 2007). 1 ml of the bacterial suspension was transferred to 100 ml glass bottles containing 19 ml ASW (with and without test compound). These suspensions were incubated at 30 °C without shaking. After 48 h, 1 ml samples were taken and the number of culturable cells was determined by plating serial dilutions on TSA (Oxoid) plates containing 2 % (w/v) NaCl. Results were expressed as numbers of viable cells present after 48 h. Each assay was repeated at least three times. The effect of the compounds on susceptibility of all the *Vibrio* strains tested towards doxycycline and chloramphenicol was determined as described previously (Brackman *et al*., 2008). Each assay was repeated at least three times. A change in MIC

was considered relevant in case of a shift of more than two doubling dilutions in either direction.

Artemia challenge tests

All experiments were performed with high quality hatching cysts of *Artemia franciscana* (EG Type, batch 6940, INVE Aquaculture). 200 mg of cysts were hydrated in 18 ml of tap water during 1 h. The procedure of Marques *et al*. (2004) was used to obtain sterile decapsulated cysts and nauplii. Challenge tests (in triplicate) were performed as described previously (Brackman *et al*., 2008).

Cytostatic activity assay

The murine (L1210) and human (CEM, HeLa) cells were seeded in a concentration of 5.0-7.5 x 10^4 cells per 200 µl in wells of a 96-well microtiter plate in the presence of serial (5-fold) dilutions of the test compound, using RPMI-1640 culture medium supplemented with 2 mM L-glutamine, 0.075 % (w/v) $NaHCO_3$, and 10 % (w/v) foetal bovine serum. After 48 h (L1210) or 72 h (CEM, HeLa), the cell numbers were determined using a Coulter Counter (Analis). The IC_{50} or 50 % inhibitory concentration of the compound represents the concentration required to inhibit cell proliferation by at least 50 %.

Statistics

The normal distribution of the data was checked using the Shapiro–Wilk test. Normally and non-normally distributed data were analyzed using an independent samples *T*-test and

218 the Mann–Whitney *U* test, respectively. Statistics were performed using SPSS software,
219 version 17.0.

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Results

Inhibition of AI-2 controlled bioluminescence

The antimicrobial activity of all compounds was evaluated against all strains used in the present study and MICs were found to be higher than 320 μM (160 μM for pyrogallol). Unless otherwise mentioned, the compounds were used in a concentration of 40 μM, which is well-below the MIC for all strains tested. Bioluminescence in a constitutively bioluminescent strain *E. coli* DH5αpBluelux was not inhibited by any of the compounds tested (Supplementary Data, Table S1). The effect on AI-2 QS was assessed using *V. harveyi* BB170. LMC-21 was the most active adenosine derivative and a concentration-dependent inhibitory effect was observed (Fig. 2). Its isomer LMC-28, which only differed in the substitution site of the methoxy group, and SC-20, a truncated ribofuranosyl analogue, also inhibited AI-2 QS (Fig. 2), but proved significantly weaker compared to LMC-21. SC-23 yielded in a significant inhibition of QS in the *V. harveyi* BB170 reporter strain only when tested in a concentration above 40 μM (Fig. 2). In addition, MCPBA, KM-03009 and pyrogallol were also able to block the AI-2 QS system (Fig. 2). All the other compounds did not result in a reduction in bioluminescence, even when used in higher concentrations (up to 160 μM).

Molecular target of the phenylpropionamidofuranosyl derivatives

To identify the molecular target of the 3'-deoxy-3'-(4-methoxyphenylpropionamido)ribofuranosyl derivatives, bioluminescence assays were conducted using several AI-2 QS mutants. No inhibitory effects were observed using the

V. harveyi JAF375 and *V. harveyi* BB886 mutant, while inhibitions were observed using the *V. harveyi* JMH597 mutant, suggesting an effect on AI-2 QS. The supernatants of *Escherichia coli* K12 treated with the compounds revealed no difference in AI-2 activity compared to the control. Further, LMC-21 blocked bioluminescence in *V. harveyi* MM30, but not in *V. harveyi* JAF553, JAF483 or BNL258, suggesting that the target is located upstream of the mutations in the AI-2 signal transduction pathway and most-likely is the LuxPQ complex in *V. harveyi*. Similar results were obtained with SC-23, LMC-28, MCPBA and KM03009, suggesting that these molecules also target LuxPQ.

Effect on protease activity and pigment production

LMC-21 significantly decreased pigment production by *V. anguillarum* LMG4411 after 48 h of growth but none of the other compounds tested was able to significantly alter pigment production (Table 2). Addition of LMC-21, MCPBA or pyrogallol resulted in a significantly decreased *V. anguillarum* LMG4411 protease activity (Table 2).

Effect on *in vitro* grown biofilms

The effect of the AI-2 QS inhibitors on the number of metabolically active cells in the biofilms of several *Vibrio* strains was evaluated using a rezasurin assay. This assay revealed no significant decrease in the number of metabolically active cells in the biofilms of the different *Vibrio* strains following treatment (Supplementary Data, Table S2). In contrast, several compounds decreased the crystal violet (CV) staining of *V. anguillarum* LMG 4411 and *V. vulnificus* LMG16867 biofilms (Table 2). However, no significant anti-biofilm effects were observed for *V. harveyi* BB120 and *V. campbellii*

LMG21363. In addition, the use of LMC-21 yielded in a minor but significant increase in CV signal for *V. cholerae* El Tor NCTC8457 (15 ± 8 % compared to the untreated control).

Effect on susceptibility of *Vibrio* spp. to stress

The effect of the different compounds on the starvation response and on the antimicrobial susceptibility of the different *Vibrio* spp. was investigated. Upon treatment with LMC-21, cell numbers significantly decreased in all *Vibrio* spp. (Table 3). Treatment with MCPBA, pyrogallol and KM-03009 reduced the number of culturable cells in some *Vibrio* spp. only (Table 3). There were no significant differences in the MIC's of all *Vibrio* strains tested for chloramphenicol and doxycycline when used alone or in combination with a QS inhibitor (Supplementary Data, Tables S3 and S4).

Effect on virulence *in vivo* and cytotoxicity

High mortality rates were observed when exposing *Artemia* to *V. harveyi* BB120, but LMC-21 was able to completely protect *Artemia* during bacterial challenge (Fig. 3). LMC-21 alone had neither an effect on *Artemia* shrimp (Fig. 3) nor on *V. harveyi* BB120 (data not shown). In addition LMC-21 was found to have IC_{50} values being ≥ 250 μM (L1210 cells) or ≥ 125 μM (CEM and HeLa cells).

Discussion

QS is an important regulator of bacterial virulence in some bacterial species. Accordingly, QS inhibition is gaining interest as a potential alternative strategy for the treatment of bacterial infections. Although LuxS appears to be omnipresent in the bacterial world, the LuxPQ signal transduction system is restricted to Vibrionales (Sun *et al*., 2004; Rezzonico & Duffy, 2008). This makes the AI-2 receptor complex of Vibrionales an interesting target for the selective control of *Vibrio* spp. QS-regulated virulence.

In this study, we not only confirmed the QS inhibitory activity of several established AI-2 QS inhibitors, but we also discovered several new inhibitors. To identify their molecular target, we evaluated the effect of the most active compound (LMC-21) on different *V. harveyi* QS mutants. Although we originally anticipated that certain adenosine analogues might disturb the biosynthesis of DPD, due to their structural similarity with S-adenosylmethionine, our data indicate that LMC-21 exerts its effect at the level of the AI-2 transduction system rather than at that of AI-2 production. For these experiments, several *V. harveyi* QS mutants with mutations in the AI-2 signal transduction system were used. *V. harveyi* JAF553 and JAF483 contain a point mutation in the *luxU* and *luxO* genes, respectively, thereby preventing phosphorelay capacity of LuxU and LuxO. *V. harveyi* BNL258 has a Tn5 insertion in the *hfq* gene, resulting in a non-functional Hfq protein. Since *V. harveyi* strains JAF553, JAF483 and BNL258 are all constitutively luminescent, a lack of inhibition of bioluminescence in one of these indicates that the inhibitor acts upstream of the mutated protein. Our compound proved incapable of blocking bioluminescence in these three QS mutants. This suggests that the target of the

312 3-(methoxyphenylpropionamido)ribofuranosyl derivatives is the upstream component of
313 the AI-2 signalling transduction pathway, LuxPQ. In addition, no effect was observed
314 when testing the compound in *V. harveyi* BB886, a mutant which lacks the LuxP receptor
315 required for AI-2 response and in *V. harveyi* JAF375, a mutant which lacks LuxQ.
316 Although several compounds inhibit the AI-2 QS system, there are few reports on QS
317 inhibitors targeting LuxPQ. Phenylboronic acids, pyrogallol derivatives and 2-(2-
318 thienylsulfonyl)ethanethioamide, previously reported to block the AI-2 QS system at the
319 level of LuxPQ (Li *et al*., 2008; Ni *et al*., 2008a; Ni *et al*., 2008b), were at best as active
320 as LMC-21. None of these compounds has been previously evaluated for its effect on AI-
321 2 related virulence. One molecule from each group of LuxPQ inhibitors was selected for
322 further experiments. LMC-21 was not only able to reduce pigment production in *V.*
323 *anguillarum* LMG4411, but also decreased protease activity in this strain. In contrast,
324 none of the established QS inhibitors targeting LuxPQ were able to block pigment
325 production or to reduce protease more than did LMC-21. In addition, LMC-21 decreased
326 the biofilm biomass of *V. anguillarum* and *V. vulnificus*, without reducing the number of
327 viable cells present in the biofilms. Pyrogallol only decreased biofilm biomass in *V.*
328 *vulnificus*, but to a higher extent than LMC-21. These data confirm the finding that
329 pigment and protease production in *V. anguillarum* and biofilm formation in *V.*
330 *anguillarum, V. vulnificus* and *V. cholerae* are (at least partially) controlled by the AI-2
331 QS system (Croxatto *et al*., 2002; Zhu *et al*., 2002; Hammer & Bassler, 2003; Lee *et al*.,
332 2007; Brackman *et al*., 2008). Mutations in the LuxR homologs of *V. anguillarum*
333 (VanT) and *V. vulnificus* (SmcR) were shown to reduce biofilm formation in these
334 species indicating that AI-2 QS may promote biofilm formation in these species

335 (Croxatto *et al*., 2002; Lee *et al*., 2007). In contrast, *V. cholerae* HapR represses the
336 expression of *vps* genes (involved in the production of exopolysaccharides) and biofilm
337 formation (Zhu *et al*., 2002; Hammer & Bassler, 2003) indicating that AI-2 QS
338 negatively influences biofilm formation in this species. However, the main QS-signalling
339 molecule in *V. cholerae* is CAI-1 and this may explain the limited impact of AI-2 QS
340 inhibitors on *V. cholerae* biofilm formation. Whether the increase in *V. cholerae* biomass,
341 due to LMC-21, would impose problems in *in vivo* situations remains to be determined.
342 In addition, *Vibrio* spp. are also known to regulate stress adaptation by means of their QS
343 system. AI-2 is capable of regulating different stress responses, including starvation in *V.*
344 *cholerae, V. vulnificus*, *V. anguillarum* and *V. angustum* (McDougald *et al*., 2001;
345 McDougald *et al.,* 2003; Larsen *et al*., 2004; Joelsson *et al*., 2007; Lee *et al*., 2007;
346 Weber *et al.,* 2008). Our data indicate that LMC-21 suppresses the QS-regulated
347 starvation response in all *Vibrio* spp. used, while the other compounds increased
348 susceptibility to starvation-associated stress conditions in some *Vibrio* spp. only and that
349 to a lesser extent than LMC-21. However, our results indicate that AI-2 inhibition in five
350 *Vibrio* spp. did not change their antimicrobial susceptibility. Of all the compounds tested,
351 LMC-21 was the most interesting one since it was clearly at least as active in inhibiting *in*
352 *vitro* virulence compared to the other active compounds tested in this study. Although a
353 decrease of virulence *in vitro* is not always linked to a decrease of virulence *in vivo*,
354 LMC-21 was shown to be a potent suppressor of *V. harveyi* BB120 virulence *in vivo*.
355 LMC-21 had no effect on *Artemia* survival as such and its lack of cytotoxicity, when used
356 at 40 μM, was confirmed using murine and human cell lines. It is interesting to notice
357 that halogenated furanones, well-documented QS inhibitors, have toxic side-effects in

concentrations comparable to those used in the present study (Defoirdt *et al.*, 2006; Janssens *et al.,* 2008).

In a preliminary search for the active pharmacophore of LMC-21, we synthesized a couple of compounds based on the phenylpropionamidofuranosyl backbone. Based on their effect on AI-2 regulated bioluminescence in *V. harveyi* BB120, we identified the most important structural elements required for achieving QS inhibition. Minor changes, e.g. removing the methoxy group from para (LMC-21) to meta position (LMC-28) or the insertion of an extra CH_2 group between the phenylpropionamido substituent and the ribose moiety (SC-23) resulted in a decreased activity. Other molecules strongly resembling LMC-21, e.g. LMC-20 (longer side chain), LMC-23 (lacking the methoxy substituted aromatic ring), LMC-27 (lacking the methoxy substitution on the aromatic ring) and IK-1, failed to inhibit the AI-2 QS system and all together point toward a specific (receptor mediated) effect. We also investigated the importance of the adenine moiety present in LMC-21 by evaluating the effect of SC-1, SC-2, SC-3 and SC-20. Only SC-20 inhibited AI-2 QS, clearly showing that the ribofuranose moiety is required for activity. In addition, these results show that, although an adenine group is not essential for activity, its presence results in more active compounds. However, the molecular interaction of these compounds with LuxPQ remains to be determined.

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625 **Tables**

626 **Table 1:** Strains used in this study. BCCM/LMG: Belgian Co-ordinated Collections of
627 Micro-organisms/Laboratory of Microbiology collection (Ghent University, Belgium);
628 HPACC: Health Protection Agency Culture collection.

Strain	Additional information	Reference or source
Vibrio harveyi		
BB120	Wild type from which strains BB170, BB886, MM30, JAF553, JAF483, BNL258, JAF375 and JMH597 are derived	[Bassler *et al*., 1997]
BB170	*luxN*::Tn5	[Bassler *et al*., 1993]
BB886	*luxPQ*::Tn5 Kan^R	[Bassler *et al*., 1994]
MM30	*luxS*::Tn5	[Surette *et al*., 1999]
JAF553	*luxU* H58A linked to Kan^R	[Freeman & Bassler, 1999a]
JAF483	*luxO* D47A linked to Kan^R	[Freeman & Bassler, 1999b]
BNL258	*hfq*::Tn5*lacZ*	[Lenz *et al*., 2004]
JAF375	*luxN*::Cm^R *luxQ*::Kan^R	[Freeman & Bassler, 1999b]
JMH597	*luxN*::Tn5 *cqsS*::Cm^R	[Defoirdt *et al*., 2006]
Vibrio anguillarum		
LMG 4411	Isolated from young sea trout (*Salmo trutta*)	BCCM/LMG
Vibrio campbellii		
LMG 21363	Isolated from *Penaeus monodon* juvenile, lymphoid organ	BCCM/LMG
Vibrio cholerae		
NCTC8457	Isolated from human, biotype El Tor	HPACC
Vibrio vulnificus		
LMG 16867	Isolated from tank water on eel	BCCM/LMG

	farm	
Escherichia coli		
DH5αpBlueLux	Strain (not producing AI-2) containing pBluelux polylinker and *luxCDABE* genes	[Brackman *et al*., 2008]
K12	AI-2 producing strain	[Ren *et al*., 2004]

Table 2: Effect of the AI-2 QS inhibitors (40 μM) on QS-regulated phenotypes. *: significantly different compared to an untreated control ($p < 0.05$; independent samples T-test).

Compound	Protease activity†	Pigment production‡	Biofilm formation§	
	V. anguillarum **LMG4411**	***V. anguillarum*** **LMG4411**	***V. anguillarum*** **LMG4411**	***V. vulnificus*** **LMG16867**
LMC-21	23 ± 3 %*	19 ± 10 %*	35 ± 11 %*	17 ± 15 %*
KM-03009	5 ± 12 %	2 ± 13 %	2 ± 22 %	5 ± 24 %
MCPBA	20 ± 2 %*	5 ± 16 %	36 ± 8 %*	18 ± 16 %*
Pyrogallol	18 ± 5 %*	10 ± 22 %	10 ± 10 %	40 ± 9%*

† % reduction in protease activity compared to an untreated control (A_{590} of 1.230 ± 0.132) (± SD)

‡ % reduction in pigment production compared to an untreated control (A_{405} of 0.480 ± 0.090)(± SD)

§% reduction in biofilm biomass (crystal violet staining) compared to an untreated control (± SD)

637 **Table 3**: Effect of the AI-2 QS inhibitors (40 μM) on QS regulated starvation response. Data are presented as numbers of viable cells

638 ($x10^8$) present after 48 h. *: significantly different from number of cells present after 48 h in the control ($p < 0.05$; Mann-Whitney U)

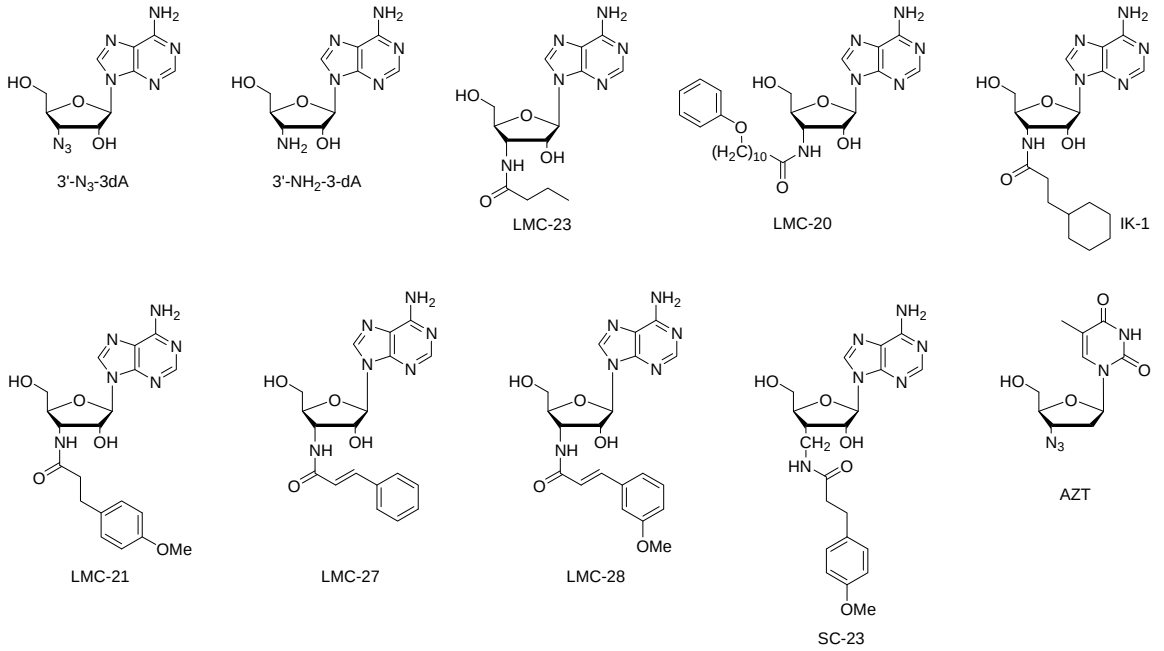
639

Compounds	**Number of viable cells ($x10^8$) (± SD)**				
	V. anguillarum **LMG4411**	***V. campbellii*** **LMG21363**	***V. cholerae*** **NCTC8457**	***V. harveyi*** **BB120**	***V. vulnificus*** **LMG16867**
Initial number of cells	1.05 ± 0.30	1.00 ± 0.23	1.16 ± 0.11	1.15 ± 0.14	1.11 ± 0.21
Control	0.77 ± 0.25	0.91 ± 0.18	1.10 ± 0.07	1.19 ± 0.45	1.09 ± 0.21
LMC-21	0.08 ± 0.07*	0.47 ± 0.09*	0.86 ± 0.12*	0.53 ± 0.43*	0.67 ± 0.01*
KM-03009	0.58 ± 0.07	0.93 ± 0.46	0.94 ± 0.45	0.85 ± 0.16*	1.11 ± 0.17
MCPBA	0.32 ± 0.17*	1.02 ± 0.32	1.24 ± 0.66	1.17 ±0.37	0.93 ± 0.11
Pyrogallol	0.21 ± 0.04*	0.56 ± 0.15*	0.91 ± 0.13	1.22 ± 0.67	1.03 ± 0.05

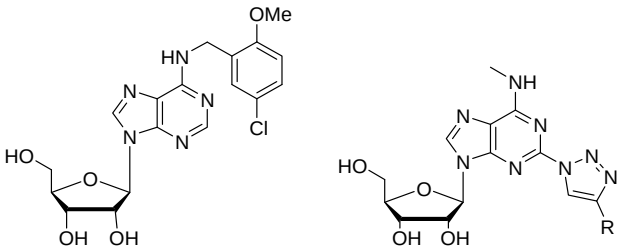
640

Fig. 1: Overview of all analogues used in this study and previously not investigated in the context of QS or biofilm inhibitory activity (A, B and C) and compounds previously only investigated for their effect on AI-2 QS (D).

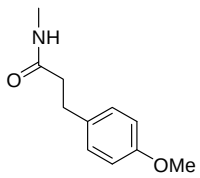
A. Sugar-modified nucleosides



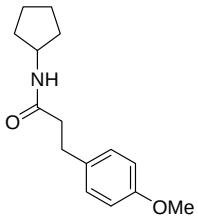
B. Base-modified nucleosides



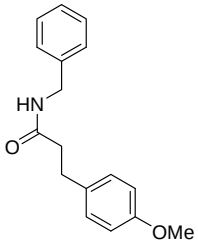
C. Simplified analogues derived from LMC-21



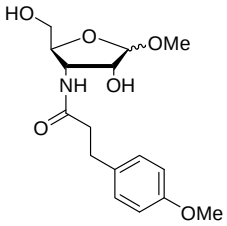
SC-1



SC-2



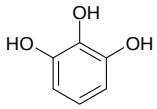
SC-3



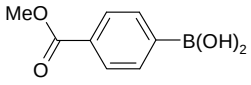
SC-20

649

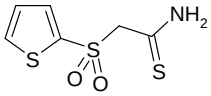
650 D. other AI-2 QSI



Pyrogallol



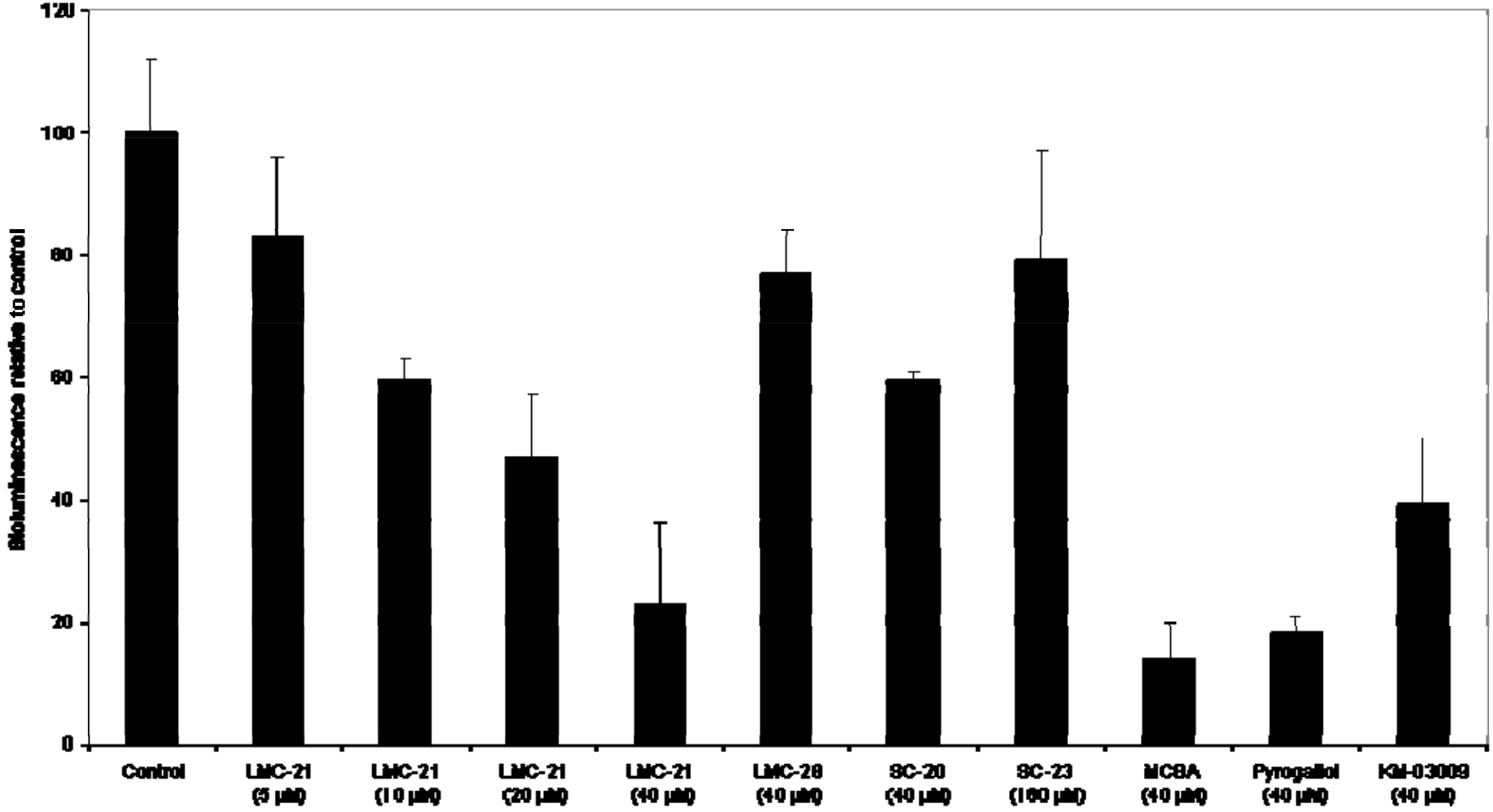
MCPBA



KM-03009

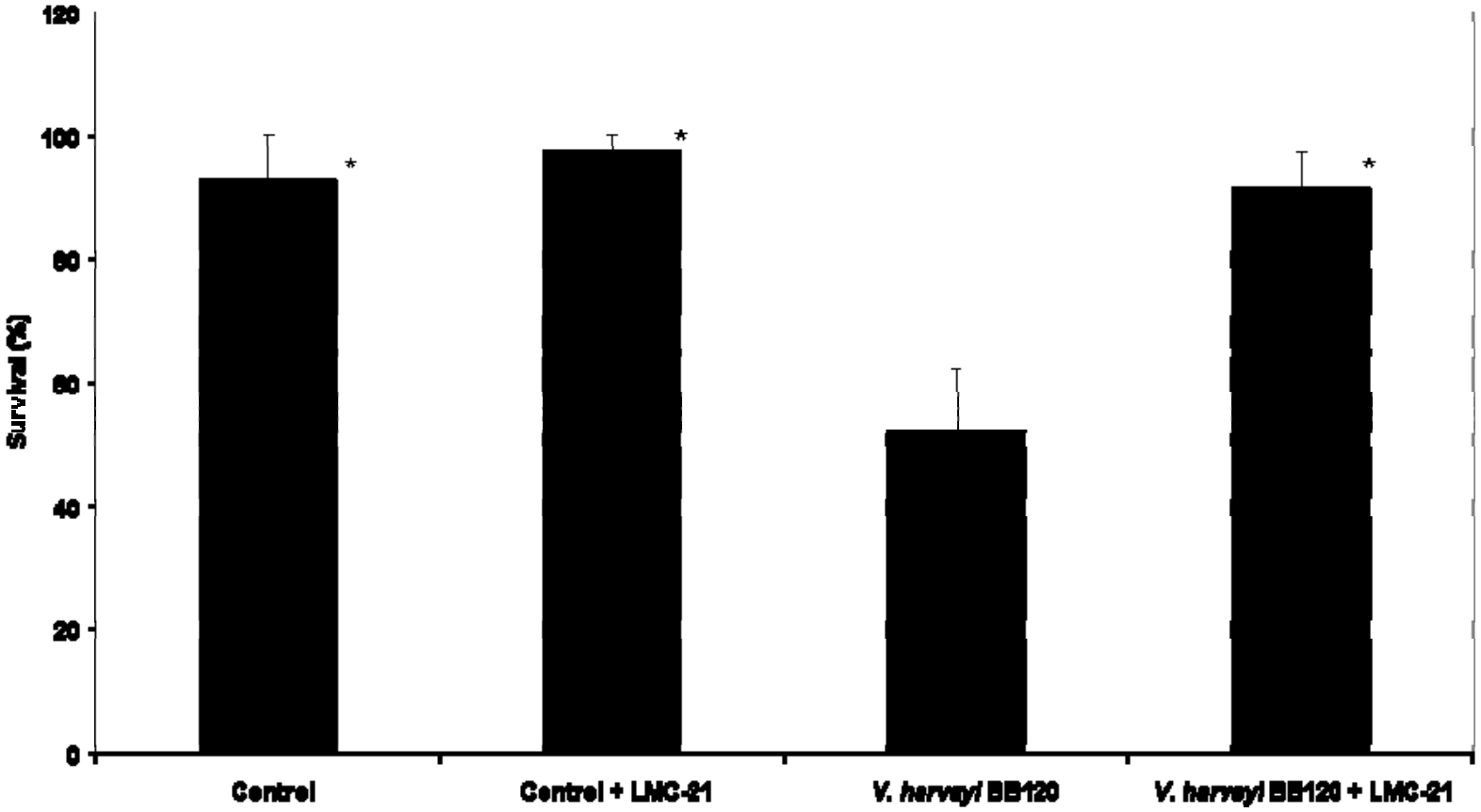
651

652 **Fig. 2:** Bioluminescence in *V. harveyi* BB170 in the absence (control) and presence of QS inhibitors. Bioluminescence measurements
653 were performed 6 h after the addition of the compounds. Bioluminescence of the control (without addition of compound) was set at
654 100 % and the responses for other samples were normalised accordingly. The error bars represent the standard deviation.
655 Bioluminesence was significantly lower than the untreated control for all compounds ($p < 0.05$).
656


Bioluminescence relative to control
120
100
80
60
40
20
0
Control
LMC-21 (5 µM)
LMC-21 (10 µM)
LMC-21 (20 µM)
LMC-21 (40 µM)
LMC-28 (40 µM)
SC-20 (40 µM)
SC-23 (100 µM)
MCBA (40 µM)
Pyrogallol (40 µM)
KM-03009 (40 µM)

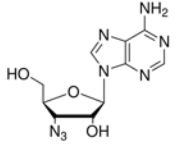
657

Fig. 3: Effect of LMC-21 on the survival of *Artemia*. Control: survival of *Artemia* without challenge with *V. harveyi* BB120 and without LMC-21. Control + LMC-21: survival of *Artemia* without challenge with *V. harveyi* BB120 in the presence of LMC-21 (40 μM). *V. harveyi* BB120: survival of *Artemia* after challenge with *V. harveyi* BB120 in the absence of LMC-21. *V. harveyi* BB120 + LMC-21: survival of *Artemia* after challenge with *V. harveyi* BB120 and treatment with LMC-21 (40 μM). *: Survival significantly different from the treatment with pathogen alone ($p < 0.001$).

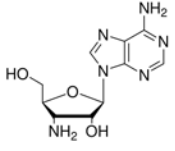

120
100
80
60
40
20
0
Survival (%)
*
*
*
Control
Control + LMC-21
V. harveyi BB120
V. harveyi BB120 + LMC-21

664

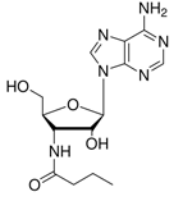
A. Sugar-modified nucleosides



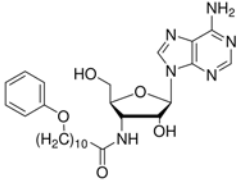
3'-N_3-3dA



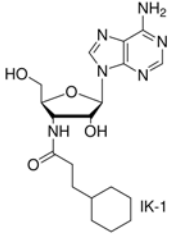
3'-NH_2-3-dA



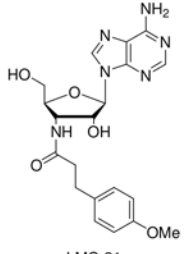
LMC-23



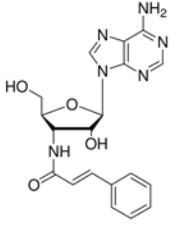
LMC-20



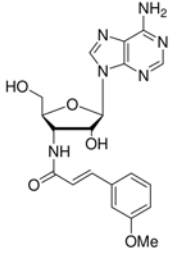
IK-1



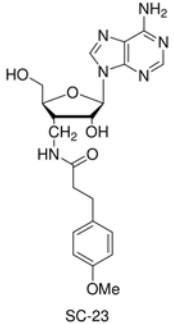
LMC-21



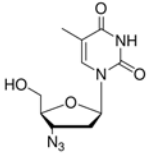
LMC-27



LMC-28

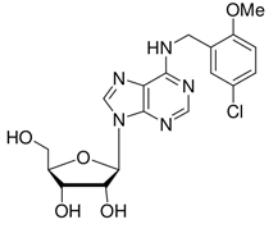


SC-23

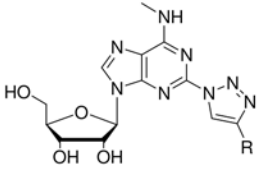


AZT

B. Base-modified nucleosides

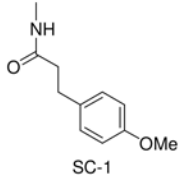


PVR-121

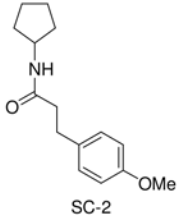


LMC 29: R = Ph
LMC 30: R = *n*-Bu
LMC 35: R = NMe_2

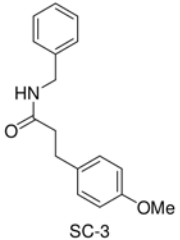
C. Simplified analogues derived from LMC-21



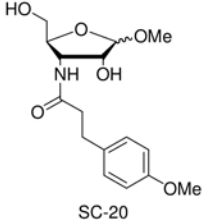
SC-1



SC-2

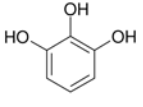


SC-3

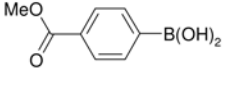


SC-20

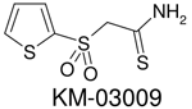
D. other AI-2 QSI



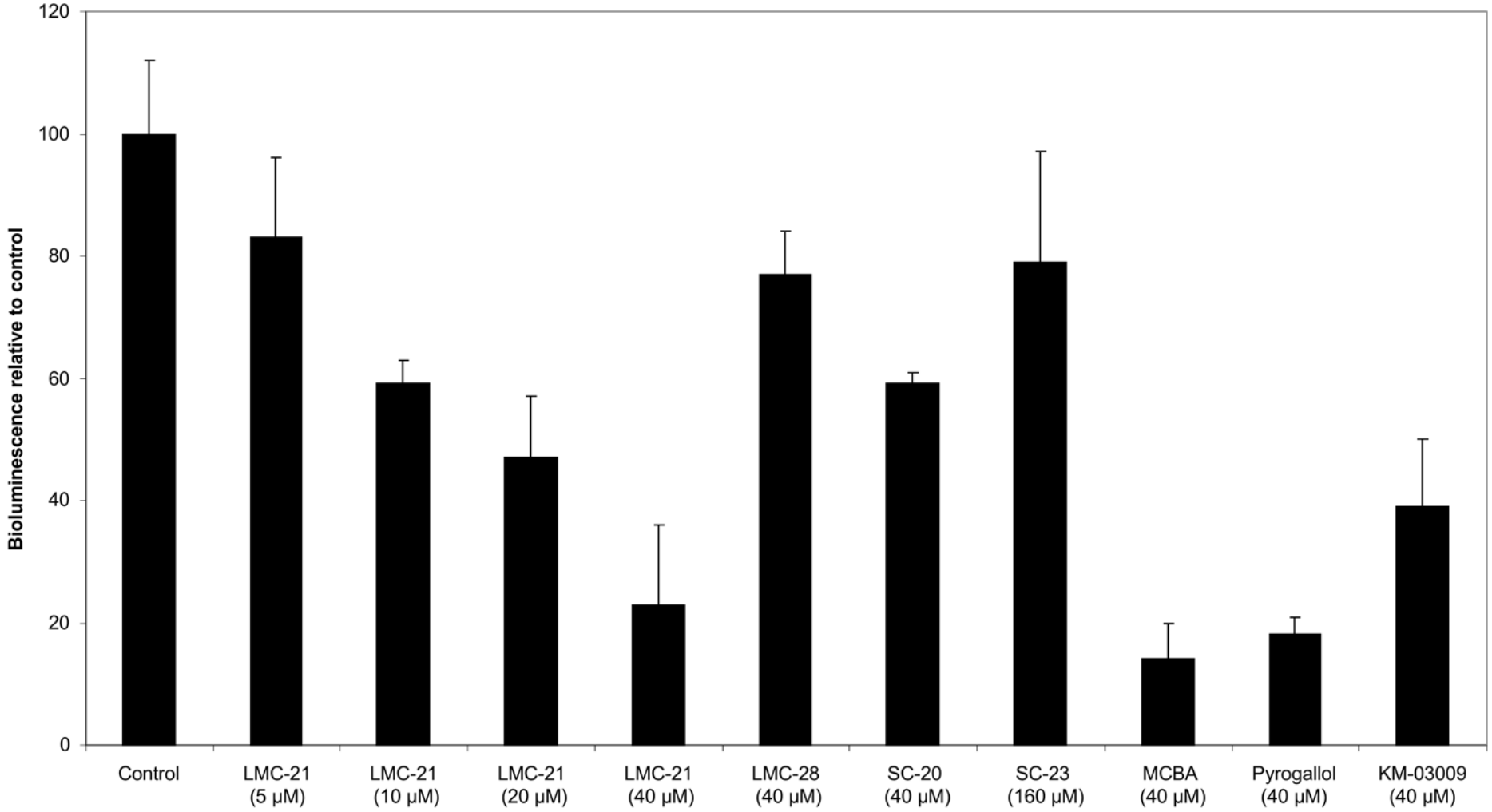
Pyrogallol

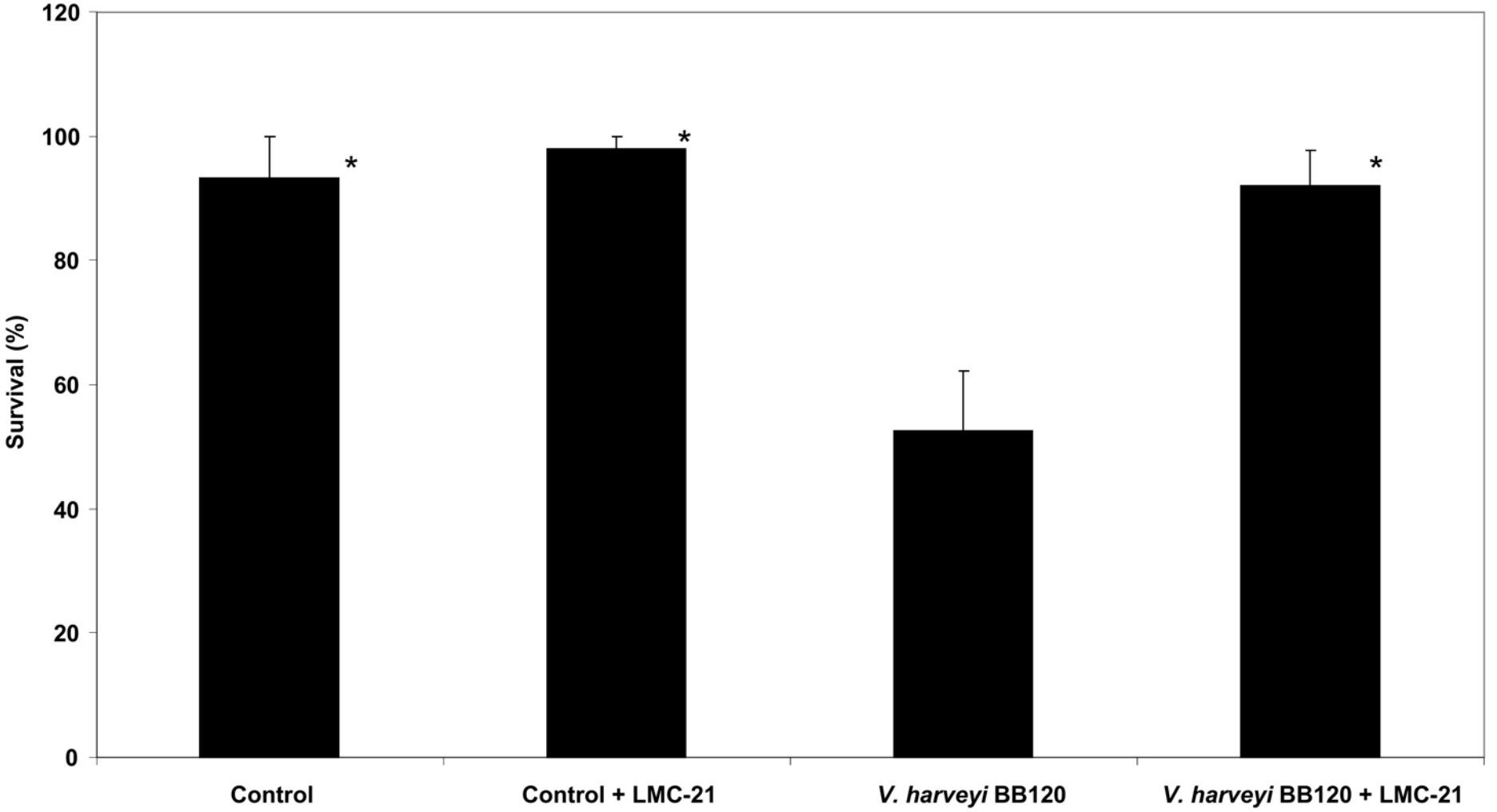


MCPBA



KM-03009


Bioluminescence relative to control
120
100
80
60
40
20
0
Control
LMC-21 (5 μM)
LMC-21 (10 μM)
LMC-21 (20 μM)
LMC-21 (40 μM)
LMC-28 (40 μM)
SC-20 (40 μM)
SC-23 (160 μM)
MCBA (40 μM)
Pyrogallol (40 μM)
KM-03009 (40 μM)


120
100
80
60
40
20
0
Survival (%)
*
*
*
Control
Control + LMC-21
V. harveyi BB120
V. harveyi BB120 + LMC-21

AI-2 quorum sensing inhibitors affect the starvation response and reduce virulence in several *Vibrio* species, most likely by interfering with LuxPQ

Gilles Brackman, Shari Celen, Kartik Baruah, Peter Bossier, Serge Van Calenbergh, Hans J Nelis, Tom Coenye

Supplementary material including:

Figure S1 : Synthesis of the amide analogues derived from 3'-NH_2-3'-dA

Figure S2 : Synthetic route followed for the synthesis of the 3'-branched-chain analogue SC-23

Figure S3 : Synthesis of SC-20

Supplementary methods : Synthesis procedures, ^{1}H- and ^{13}C nuclear magnetic resonance spectroscopy and exact mass measurements of compounds synthesised during the present study

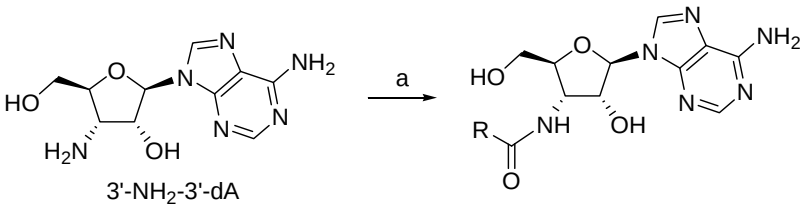
Supplementary Table 1: Lack of effect of various compounds on the constitutive bioluminescence of *E. coli* DH5αpBlueLux. Expressed as % (mean±standard deviation) of luminescence in control without compound.

Supplementary Table 2 : Relative number (expressed as %) metabolically active cells in biofilms, compared to untreated controls (mean±standard deviation). Data are based on resazurin viability staining.

Supplementary Table 3 : MIC (μg/ml) for chloramphenicol when used alone or in combination with QSI.

Supplementary Table 4 : MIC (μg/ml) for doxycycline when used alone or in combination with QSI.

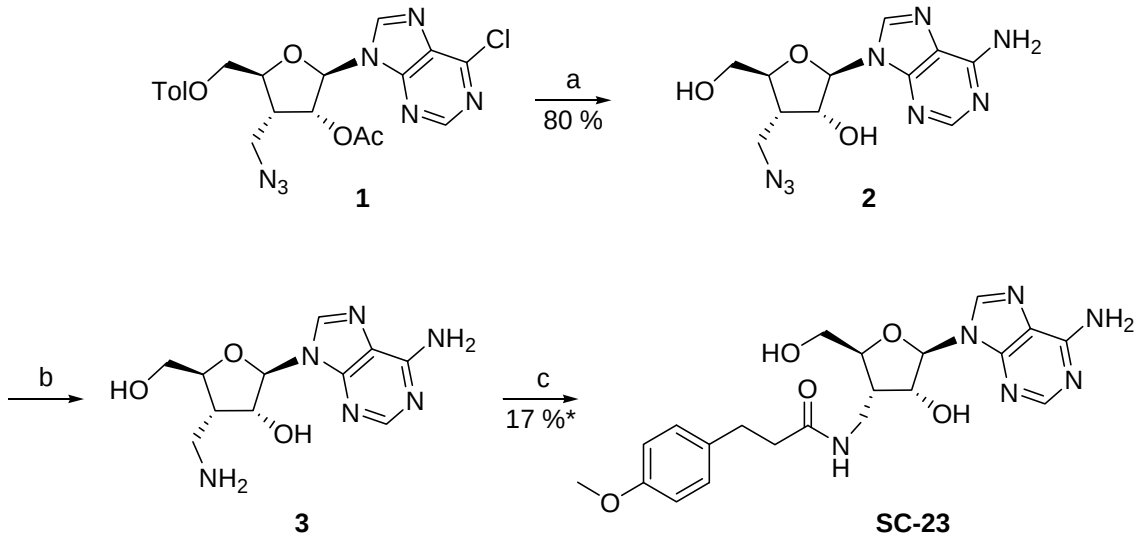
Figure S1 : Synthesis of the amide analogues derived from 3'-NH_2-3'-dA



3'-NH_2-3'-dA

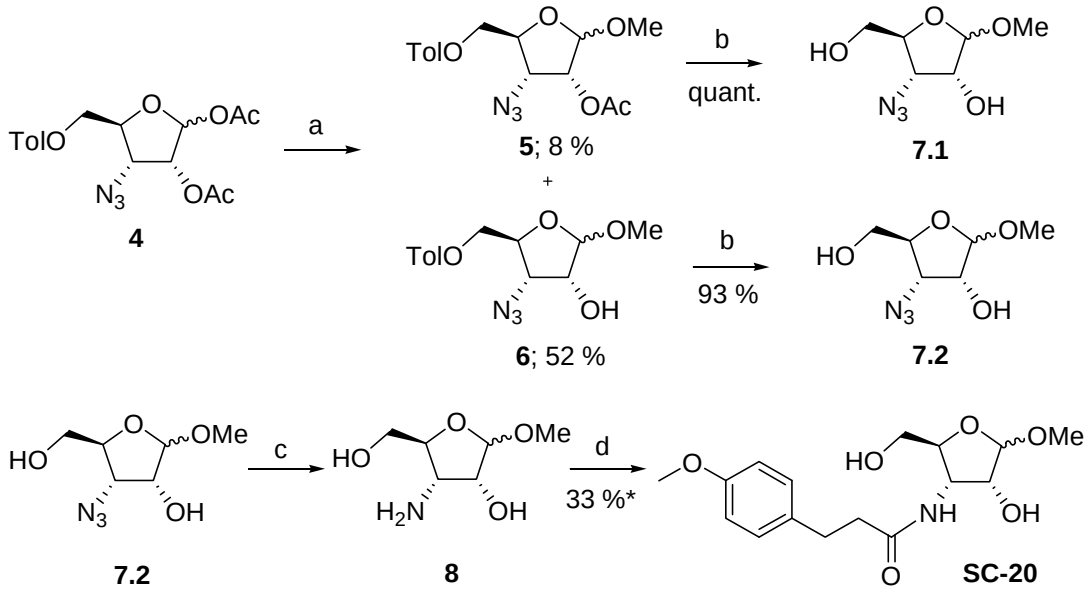
a) NHS, DCC or DIC, RCOOH, DMF/CH_2Cl_2, rt, 5 days

Figure S2 : Synthetic route followed for the synthesis of the 3'-branched-chain analogue SC-23



a) NH_3 in EtOH (2 M), 105 °C, 45 h, NH_3 in MeOH (7 M), rt, 16 h, then: NaOMe, MeOH, rt, 20h; b) PPh_3, THF, pyridine, rt, 8 h, then H_2O, rt, 16 h; c) [3-(4-methoxyphenyl)propanoic acid, HCTU, dipea, DMF, rt, 1 h], DMF, rt, 18 h; *yield over 2 steps.

Figure S3 : Synthesis of SC-20



a) $SnCl_4$, DCM, 0 °C, 15 min, MeOH, 0 °C, 1 h, 0 °C → rt, 3 h; b) NH_3 in MeOH (7 N), rt, 43 h; c) PPh_3, pyridine, rt, 8 h, H_2O, rt, 16 h; d) [3-(4-methoxyphenyl)propanoic acid, HCTU, dipea, DMF, rt, 1 h], DMF, rt, 23 h; * yield over 2 steps.

Supplementary methods : Synthesis procedures, ^{1}H- and ^{13}C nuclear magnetic resonance spectroscopy and exact mass measurements of compounds synthesised during the present study

1. Synthesis of 3'-deoxy-3'-amidoadenosines (LMC-23, LMC-20, IK-1, LMC-21, LMC-27, LMC-28), exemplified for LMC-21

To a solution of 3'-NH_2-3'-dA (11.32 mg; 42.5 µmol) in DCM (1.2 mL) and DMF (0.5 mL), NHS (6.97 mg; 60.6 µmol) and DIC (0.01 mL; 64.2 µmol) were added. After stirring for 45 min at rt under N_2-atmosphere, TLC (DCM/0.6 N NH_3 in MeOH 4:1) indicated the reaction to be incomplete. To increase the solubility of the starting material the DCM was largely evaporated and DMF (0.5 mL) was added, followed by an additional amount of DIC (0.01 mL). After 5 days the starting amine was completely converted. The solvents were evaporated and the residue purified by column chromatography (DCM/0.6 N NH_3 in MeOH 93:7) to afford the title compound as a light yellow oil (10.16 mg; 56 %).

^{1}H-NMR (300 MHz, $C_5D_5N - d_5$): δ 8.99 (s, 1H, *arom. H*); 8.72 (br.s, 1H, -CO-N*H*-); 8.63 (s, 1H, *arom. H*); 8.55 (br.s, 1H, -O*H*); 8.32 (br.s, 2H, -NH_2); 7.23 (d, *J* = 8.7 Hz, 2H, *arom. H*); 6.92 (d, *J* = 8.7 Hz, 2H, *arom. H*); 6.67 (d, *J* = 2.3 Hz, 1 H, *H-1'*); 5.55 – 5.45 (m, 1H, *H-3'*); 5.21 (dd, *J* = 2.2 and 5.4 Hz, 1H, *H-2'*); 5.02 (br.s, 1H, -O*H*); 4.66 (dt, *J* = 2.4 and 7.9 Hz, 1H, *H-4'*); 4.37 (dd, *J* = 2.1 and 12.3 Hz, 1H, *H-5'A*); 4.21 (dd, *J* = 3.2 and 12.6 Hz, 1H, *H-5'B*); 3.63 (s, 3H, CH_3-O-); 3.22 − 3.00 (m, 2H, -CH_2-CO-NH-); 2.90 − 2.72 (m, 2H, -Ph-CH_2-); ^{13}C-NMR (75 MHz, $C_5D_5N - d_5$): δ 173.83; 159.14; 158.12; 154.08; 140.05; 134.45; 130.39; 121.66; 114.91; 92.23; 85.65; 75. 61; 62.39; 55.70; 52.07; 39.01; 31.88; HRMS (ESI-MS): m/z: calcd: 429.1881 [M+1]; found 429.1891 [M+1].

2. Synthesis of 3'-deoxy-3'-*C*-(3-(4-methoxyphenyl)propionamidomethyl)adenosine (SC-23)

*2.1. Synthesis of 9-(3-C-azidomethyl-3-deoxy-β-D-ribofuranosyl)adenine (**2**)*

1 (398.67 mg; 0.82 mmol), dissolved in a 2 M solution of NH_3 in EtOH (5 mL), was stirred for 22 h in a sealed tube at 105 °C. After that time TLC (EtOAc) indicated the incomplete deprotection of **1**. Addition of an extra amount of NH_3 in EtOH (2 M, 5 mL) and NH_3 in MeOH (7 N, 5 mL) didn't solve this issue. After evaporation of the reaction mixture, a solution of NaOMe (30%) in MeOH (20 mL) was added. After stirring for 16h at rt, an additional amount of the ethanolic NaOMe solution (20 mL) was added and the reaction temperature was raised to 40 °C. After stirring for an additional 4h, the reaction was quenched by addition of H_2O. The title compound was obtained as a white solid (200.44 mg; 0.65 mmol; 80%) after purification by column chromatography (DCM/MeOH 9:1).

^{1}H-NMR (300 MHz, $(CD_3)_2SO - d_6$): δ 8.41 (s, 1H, *arom. H*); 8.15 (s, 1H, *arom. H*); 7.29 (s, 2H, -NH_2); 6.05 (d, *J* = 4.9 Hz, 1H, *H-1'*); 5.93 (d, *J* = 2.2 Hz, 1H, 2'-O*H*); 5.22 (t, *J* = 5.5 Hz, 1H, 5'-O*H*); 4.61 − 4.57 (m, 1H, *H-2'*); 4.02 (dt, *J* = 3.1 and 8.6 Hz, 1H, *H-4'*); 3.79 − 3.43 (m, 4H, *H-5'* and *H-6'*); 2.70 − 2.60 (m, 1H, *H-3'*); ^{13}C-NMR (75 MHz, $(CD_3)_2SO - d_6$): δ 156.75; 153.13; 149.43; 139.71; 119.80; 91.06; 83.63; 75.36; 62.20; 48.21; 42.18.

*2.2. Synthesis of 9-(3-C-aminomethyl-3-deoxy-β-D-ribofuranosyl)adenine (**3**)*

To a solution of **2** (192.50 mg; 0.63 mmol) in dry THF (9 mL) triphenylphosphine (347 mg; 1.32 mmol) was added. To increase the solubility of **2** an additional amount of fry pyridine (4 mL) was added and after stirring for 8h at rt all starting material was converted.

Subsequently, H_2O (0.5 mL) was added and the reaction was stirred for 16h. The solvents were evaporated and the crude amine **3** was used without further purification.

2.3. Synthesis of ***SC-23***

A mixture of 3-(4-methoxyphenyl)propanoic acid (137.68 mg; 0.76 mmol) and HCTU (393.60 mg; 0.95 mmol) in dry DMF (5 mL) containing dipea (330 µL; 1.89 mmol) was stirred for 60 min at rt under N_2-atmosphere. After that time crude **3**, as obtained in 2.2 and dissolved in dry DMF (8 mL), was added. TLC (DCM/0.7 N NH_3 in MeOH 3:1) showed that the reaction was completed after 18h. Purification by column chromatography (DCM/0.7 N NH_3 in MeOH 9:1) afforded **SC-23** (47.56 mg; 0.11 mmol; 17 %), which was further purified upon precipitation from a mixture of MeOH and diethyl ether to afford white solid material.

^{1}H-NMR (300 MHz, C_5D_5N – *d_5*): δ 8.99 (s, 1H, *H-8*); 8.86 (br.s, 1H, -CO-N*H*); 8.66 (s, 1H, *H-2*); 8.28 (s, 2H, -NH_2); 7.70 (br.s, 1H, 2'-O*H*); 7.18 (d, *J* = 8.8 Hz, 2H, *arom. H*); 6.83 (d, *J* = 8.7 Hz, 2H, *arom. H*); 6.69 (s, 1H, *H-1'*); 5.01 (br.s, 2H, *H-2'* and 5'-O*H*); 4.61 (d, *J* = 9.4 Hz, 1H, *H-4'*); 4.37 (dd, *J* = 2.3 and 12.2 Hz, 1H, *H-5'*); 4.22 − 4.02 (m, 2H, *H-5'* and *H-6'*); 3.76 (dt, *J* = 5.3 and 13.4 Hz, 1H, *H-6'*); 3.60 (s, 3H, -OCH_3); 3.27 (sep, *J* = 4.68 Hz, 1H , *H-3'*); 3.07 (t, *J* = 7.4 Hz, 2H, -CH_2-CO-NH-); 2.67 (t, *J* = 7.4 Hz, 2H, -Ph-CH_2-); ^{13}C-NMR (75 MHz, C_5D_5N – *d_5*): δ 173.90; 158.85; 157.81; 153.75; 139.80. 134.07; 130.08; 121.39; 114.56; 92.47; 84.89; 77.35; 62.41; 55.41; 43.70; 38.95; 36.58; 31.59; HRMS (ESI-MS): m/z: calcd: 443.2037 [M+1]; found = 443.2025 [M+1].

3. General procedure for the synthesis of amides SC-1, SC-2 and SC-3

To a mixture of 3-(4-methoxyphenyl)propanoic acid (1.0 g; 5.55 mmol) and EDC (1.6 g; 8.33 mmol) in dry THF (20 mL) were added 2.5 mL of TEA and 5 mL of a 2 M solution of the appropriate amine in THF. After stirring for 16h at rt, EtOAc (50 mL) was added and the organic phase was washed successively with an aqueous HCl solution (1 N; 50 mL; 2x), a saturated $NaHCO_3$ solution (50 mL; 2x) and brine (50 mL). The organic phase was dried over Na_2SO_4 and evaporated. The residue could be purified by crystallization.

*3.1. 3-(4-methoxyphenyl)-N-methylpropanamide (****SC-1****)*

The title compound was crystallized from a mixture of isopropyl ether and heptanes to afford a first crop of colorless crystal needles (0.132 g; 0.68 mmol; 12 %).

^{1}H-NMR (300 MHz, $(CD_3)_2CO$ – *d_6*): δ 7.12 (d, *J* = 8.8 Hz, 2H, *arom. H*) ; 6.92 (br.s, 1H, -CO-N*H*-); 6.81 (d, *J* = 8.5 Hz, 2H, *arom. H*); 3.75 (s, 3H, -OCH_3); 2.82 (t, *J* = 8.2 Hz, 2H, -CH_2-CO-NH-); 2.66 (d, *J* = 4.7 Hz, 3H, -CO-NH-CH_3); 2.38 (t, *J* = 7.8 Hz, 2H, -Ph-CH_2-); ^{13}C-NMR (75 MHz, $(CD_3)_2CO$ – *d_6*): δ 171.95; 158.32; 133.81; 129.40; 113.85; 54.74; 38.06; 30.81; 25.33; HRMS (ESI-MS): m/z: calcd: 194.1176 [M+1]; found 194.1173 [M+1].

*3.2. N-cyclopentyl-3-(4-methoxyphenyl)propanamide (****SC-2****)*

The title compound was crystallized from isopropyl ether to afford a first crop of colorless crystal needles (0.418 g; 1.69 mmol; 30 %).

^{1}H-NMR (300 MHz, $(CD_3)_2CO$ – *d_6*): δ 7.11 (d, *J* = 8.8 Hz, 2H, *arom. H*); 6.91 (br.s, 1H, -CO-N*H*-); 6.81 (d, *J* = 8.8 Hz, 2H, *arom. H*); 4.12 (sxt, *J* = 6.8 Hz, 1H, *cyclopentyl-H*); 3.74 (s, 3H, -OCH_3); 2.82 (t, *J* = 8.2 Hz, 2H, -CH_2-CO-NH-); 2.35 (t, *J* = 7.8 Hz, 2H, -Ph-CH_2-); 1.90 − 1.76 (m, 2H, *cyclopentyl-H*); 1.68 – 1.46 (m, 4H, *cyclopentyl-H*); 1.43 – 1.28 (m, 2H, *cyclopentyl-H*); ^{13}C-NMR (75 MHz, $(CD_3)_2CO$ – *d_6*): δ 171.01; 158.32; 133.81; 129.43; 113.82; 54.75; 50.89; 38.21; 32.74; 30.93; 23.67; HRMS (ESI-MS): m/z: calcd: 248.1645 [M+1]; found 248.1632 [M+1].

*3.2. N-benzyl-3-(4-methoxyphenyl)propanamide (**SC-3**)*

The title compound was crystallized from isopropyl ether to afford a first crop of colorless crystal needles (0.643 g; 2.39 mmol; 43 %).

^{1}H-NMR (300 MHz, $(CD_3)_2CO - d_6$): δ 7.42 (br.s, 1H, -CO-N*H*-); 7.30 − 7.10 (m, 7H, *benz. H* and *arom. H*); 6.82 (d, *J* = 8.8 Hz, 2H, *arom. H*); 4.36 (d, *J* = 6.2 Hz, 2H, -CO-NH-C*H*$_2$-Ph); 3.76 (s, 3H, -OC*H*$_3$); 2.87 (t, *J* = 7.6 Hz, 2H, -C*H*$_2$-CO-NH-); 2.49 (t, *J* = 7.6 Hz, 2H, -Ph-C*H*$_2$-); ^{13}C-NMR (75 MHz, $(CD_3)_2CO - d_6$): δ 171.50; 158.37; 140.00; 133.64; 129.54; 128.42; 127.59; 126.90; 113.868; 54.76; 42.69; 38.14; 30.84; HRMS (ESI-MS): m/z: calcd: 270.1489 [M+1]; found 270.1502 [M+1].

4. Synthesis of 3-deoxy-3-(3-(4-methoxyphenyl)propionamido)-1-*O*-methyl-D-ribofuranose (SC-20)

*4.1. Synthesis of 2-O-acetyl-3-azido-3-deoxy-1-O-methyl-5-O-toluoyl-D-ribofuranose (**5**) and 3-azido-3-deoxy-1-O-methyl-5-O-toluoyl-D-ribofuranose (**6**)*

To a round-bottom flask supplied with flame-dried molecular sieves and kept under N_2-atmosphere, was added a solution of **4** (502.85 mg; 1.33 mmol) in dry DCM (15 mL). The solution was cooled in an ice-bath and $SnCl_4$ (320 µL; 2.66 mmol) was added. After stirring for 15 min, dry MeOH (175 µL; 4.26 mmol) was dripped in the reaction mixture. After stirring for 1h at 0 °C the reaction mixture was allowed to come to rt and stirred for another 3h. After that time TLC (hexane/EtOAc 3:1) showed the disappearance of the starting material and the formation of two new products. The reaction mixture was diluted with DCM (150 mL), washed with a saturated $NaHCO_3$ solution (150 mL, 2x) and the aqueous layer was extracted with EtOAc (100 mL). The combined organic layers were then washed with brine (150 mL), dried over Na_2SO_4, and evaporated. Compounds **5** and **6** were obtained as light yellow oils (respectively 39.4 mg; 8 % and 212 mg; 52 %) after purification with column chromatography (hexane/EtOAc 9:1 → 3:1).

5: ^{1}H-NMR (300 MHz, $(CD_3)_2CO - d_6$): δ 7.98 (d, *J* = 8.3 Hz, 2H, *arom. H*), 7.34 (d, *J* = 8.0 Hz, 2H, *arom. H*); 5.27 (d, *J* = 4.79 Hz, 1H, *H-2*); 4.93 (s, 1H, *H-1*); 4.58 (dd, *J* = 3.8 and 12.13 Hz, 1H, *H-5*); 4.47 − 4.38 (m, 2H, *H-5* and *H-3*); 4.31 (ddd, *J* = 3.83 and 4.15 and 7.98 Hz, 1H, *H-4*); 3.30 (s, 3H, -OC*H*$_3$); 2.42 (s, 3H, -CO-C*H*$_3$); 2.13 (s, 3H, C*H*$_3$-Ph-); ^{13}C-NMR (75 MHz, $(CD_3)_2CO - d_6$): δ 169.49; 165.87; 144.16; 129.78; 129.37; 127.54; 106.33; 79.22; 76.40; 64.24; 61.04; 54.52; 20.90; 19.88.

6: ^{1}H-NMR (300 MHz, $(CD_3)_2CO - d_6$): δ 7.93 (d, *J* = 8.3 Hz, 2H, *arom. H*); 7.33 (d, *J* = 8.0 Hz, 2H, *arom. H*); 4.93 (d, *J* = 4.17 Hz, 1H, *H-1*); 4.54 − 4.36 (m, 3H, *H-2* and *H-4* and *2-OH*); 4.22 (dd, *J* = 4.16 and 8.33 Hz, 1H, *H-5*); 4.13 (dd, *J* = 4.16 and 8.33 Hz, 1H, *H-5*); 3.96 (d, *J* = 9.3 Hz, 1H, *H-3*); 3.40 (s, 3H, -OC*H*$_3$); 2.41 (s, 3H, C*H*$_3$-Ph-); ^{13}C-NMR (75 MHz, $(CD_3)_2CO - d_6$): δ 165.83; 144.16; 129.70; 129.44; 127.51; 102.81; 79.43; 73.34; 64.53; 61.47; 54.73; 20.91.

*4.2. Synthesis of **7.1** and **7.2**, the two anomers of 3-azido-3-deoxy-1-O-methyl-D-ribofuranose*

Compounds **5** (39.4 mg; 0.11 mmol) and **6** (212 mg; 0.69 mmol) were separately treated with a methanolic solution of NH_3 (7 N, respectively 18 and 25 mL) at rt. After 43h TLC (hexane/EtAOc 3:1) showed that both reactions were completed. After removal of the volatiles by evaporation, both residues were purified by column chromatography (hexane/acetone 7:3 for **7.1** and hexane/acetone 13:7 for **7.2**).

7.1: ^{1}H-NMR (300 MHz, $(CD_3)_2CO - d_6$): δ 4.80 (br.s, 1H, -O*H*); 4.78 (s, 1H, *H-1*); 4.20 (br.s, 1H, *H-2*); 4.11 (dt, *J* = 3.8 and 8.9 Hz, 1H, *H-4*); 3.86 − 3.76 (m, 2H, -O*H* and *H-*

3); 3.65 (br.s, 2H, *H-5*); 3.30 (s, 3H, -OCH_3); ^{13}C-NMR (75 MHz, $(CD_3)_2CO$ – d_6): 108.71; 81.24; 76.13; 63.29; 62.36; 54.33.

7.2: ^{1}H-NMR (300 MHz, $(CD_3)_2CO$ – d_6): δ 4.84 (d, J = 4.1 Hz, 1H, *H-1*); 4.33 − 4.23 (m, 1H, *H-2*); 3.93 (m, 3H, -O*H* and *H-3* and *H-4* of *H-5A*); 3.79 (d, J = 9.77 Hz, 1H, -O*H*); 3.64 (dd, J = 3.6 and 5.5 Hz, 2H, *H-5B* and *H-5B* of *H-5B* and *H-4*); 3.37 (s, 3H, -OCH_3); ^{13}C-NMR (75 MHz, $(CD_3)_2CO$ – d_6):102.71; 82.44;73.39; 62.40; 61.46; 54.51.

*4.3. Synthesis of 3-amino-3-deoxy-1-O-methyl-D-ribofuranose (**8**)*

To a solution of **7.2** (121.70 mg; 0.64 mmol) in dry pyridine (6 mL) triphenylphosphine (363.80 mg; 1.39 mmol) was added and the mixture was stirred for 8h at rt under N_2-atmosphere. After that time the starting material was completely consumed (TLC: DCM/0.7 N NH_3 in MeOH 9:1). Subsequently, H_2O (0.5 mL) was added and after stirring for 16h, the solvents were removed and the residue was used in the next step without further purification.

*4.4. Synthesis of 3-deoxy-3-(3-(4-methoxyphenyl)propionamido)-1-O-methyl-D-ribofuranose (**SC-20**)*

After incubating a mixture of 3-(4-methoxyphenyl)propanoic acid (145.39 mg; 0.81 mmol), HCTU (397.54 mg; 0.96 mmol) and dipea (330 µL; 1.89 mmol) in dry DMF (5 mL) for 60 min at rt under N_2-atmosphere, a solution of the residue obtained in 4.3 in dry DMF (4 mL) was added. After stirring for 23h TLC (DCM/0.7 N NH_3 in MeOH 3:1) demonstrated complete reaction. Following purification by column chromatography (DCM/0.7 N NH_3 in MeOH 19:1) and crystallization of a contaminant from a mixture of EtOAc and isopropyl ether, a second chromatographic purification (hexane/acetone 1:1) afforded a residue that was dissolved in EtOAc. This solution was washed successively with a HCl solution (0.5 N, 15 mL, 3x) and brine (50 mL), dried over Na_2SO_4, and evaporated to afford pure **SC-20** as a colorless viscous oil (67.40 mg; 0.21 mmol; 33 %).

^{1}H-NMR (300 MHz, $(CD_3)_2CO$ – d_6): δ 7.14 (d, J = 8.8 Hz, 2H, *arom. H*); 6.90 (br.s, 1H, -CO-N*H*-); 6.83 (d, J = 8.8 Hz, 2H, *arom. H*); 4.86 (d, J = 4.1 Hz, 1H, *H-1*); 4.22 − 4.14 (m, 2H, *H-2* and *H-3*); 4.05 (dd, J = 5.3 and 7.0 Hz, 1H, *5-OH*); 3.85 (d, J = 7.0 Hz, 1H, *2-OH*); 3.77 − 3.72 (m, 4H, -OCH_3 and *H-4*); 3.64 − 3.51 (m, 2H, *H-5*); 3.35 (s, 3H, -OCH_3); 2.84 (dd, J = 6.4 and 8.8 Hz, 2H, -CH_2-CO-); 2.51 (dd, J = 6.8 and 8.6 Hz, 2H, -Ph-CH_2-); ^{13}C-NMR (75 MHz, $(CD_3)_2CO$ – d_6): δ 173.22; 159.06; 134.10; 130.13; 114.56; 103.81; 85.13; 71.58; 63.36; 55.43; 55.18; 51.93; 38.62; 31.45; HRMS (ESI-MS): m/z: calcd: 326.1604 [M+1]; found 326.1598 [M+1].

5. Elemental Analysis

	Calculated			Found		
	C	**H**	**N**	**C**	**H**	**N**
LMC 23	49.99	5.99	24.99	50.22	6.13	24.33
LMC-20	61.58	7.27	15.96	61.02	7.38	15.80
IK-1	56.42	6.98	20.78	56.80	6.75	20.44
LMC-21	56.07	5.65	19.62	56.00	5.83	19.29
LMC-27	57.57	5.09	21.20	57.90	5.39	20.97
LMC-28	56.33	5.20	19.71	56.33	5.29	19.62
SC-23	57.00	5.92	18.99	57.32	5.90	18.71
PVR-21	51.25	4.78	16.60	51.10	4.88	16.15
LMC-29	53.77	4.75	26.40	53.67	4.89	26.08

LMC-30	50.49	5.98	27.71	50.69	6.08	27.41
LMC-35	47.40	5.72	31.09	47.55	5.64	30.41
SC-1	68.37	7.82	7.25	68.44	7.91	7.33
SC-2	72.84	8.56	5.66	72.99	8.69	5.68
SC-3	75.81	7.11	5.20	76.03	7.19	5.09
SC-20	59.06	7.13	4.31	Not available		

Supplementary Table 1: Lack of effect of various compounds on the constitutive bioluminescence of *E. coli* DH5αpBlueLux. Expressed as % (mean±standard deviation) of luminescence in control without compound.

Compound	Luminescence in *E. coli* DH5α pBluelux
KM-03009 (40 μM)	97 ± 8 %
LMC-21 (40 μM)	98 ± 4 %
LMC-28 (40 μM)	101 ± 6 %
MCPBA (40 μM)	96 ± 11 %
Pyrogallol (40 μM)	95 ± 14 %
SC-23 (160 μM)	103 ± 7
SC-20 (40 μM)	102 ± 11 %

Supplementary Table 2 : Relative number (expressed as %) metabolically active cells in biofilms, compared to untreated controls (mean±standard deviation). Data are based on resazurin viability staining.

	V. anguillarum LMG4411	*V. campbellii* LMG21363	*V. cholerae* NCTC8457	*V. harveyi* BB120	*V. vulnificus* LMG16867
Control	100 ± 23	100 ± 18	100 ± 12	100 ± 11	100 ± 19
LMC-21	90 ± 11	99 ± 10	102 ± 14	104 ± 8	97 ± 8
KM-03009	101 ± 14	104 ± 12	104 ± 16	104 ± 9	105 ± 13
MCPBA	101 ± 26	93 ± 9	93 ± 22	103 ± 13	92 ± 22
Pyrogallol	91 ± 23	96 ± 18	99 ± 18	106 ± 12	95 ± 18

Supplementary Table 3 : MIC (μg/ml) for chloramphenicol when used alone or in combination with QSI.

	V. anguillarum LMG4411	*V. campbellii* LMG21363	*V. cholerae* NCTC8457	*V. harveyi* BB120	*V. vulnificus* LMG16867
Control	1	2	1	2	1
LMC-21	1	2	1	2	1
KM-03009	1	2	1	2	1
MCPBA	1	2	1	2	1
Pyrogallol	1	2	1	2	1

Supplementary Table 4 : MIC (μg/ml) for doxycycline when used alone or in combination with QSI.

	V. anguillarum LMG4411	*V. campbellii* LMG21363	*V. cholerae* NCTC8457	*V. harveyi* BB120	*V. vulnificus* LMG16867
Control	64	64	32	64	8
LMC-21	64	64	32	64	8
KM-03009	64	64	32	64	8
MCPBA	64	64	32	64	8
Pyrogallol	64	64	32	64	8